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ORIGINAL ARTICLE

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# Systemic induction of volatile release in cotton: How specific is the signal to herbivory?

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**Abstract** Plants attacked by herbivorous insects release chemical signals that attract natural enemies of the herbivores to the damaged plants. Feeding of *Spodoptera exigua* larvae on the lower leaves of cotton (*Gossypium hirsutum* L.) for multiple feeding periods of 9–12 h with a 12 h, interval in between when the caterpillars are removed overnight, will induce a systemic release of volatile compounds that is comparable to the volatiles released in response to continuous feeding damage on the lower leaves for several days. The systemic volatile release in response to herbivory can be mimicked by mechanically damaging the lower leaves and applying caterpillar oral secretion to the injured leaves over 4 days. Cotton plants that are only mechanically damaged systemically release significantly less β-pinene, myrcene, (*Z*)-3-hexenyl acetate, (*E*)-β-farnesene and (*E,E*)-α-farnesene after 4 days compared to plants damaged mechanically with application of caterpillar regurgitant. However, multiple 9–12 h mechanical damage alone induces a significantly higher systemic release of (*Z*)-3-hexenyl acetate, myrcene, (*E*)-β-ocimene, and (*E*)-β-farnesene after 4 days compared to undamaged control plants. This indicates that multiple mechanical damage alone cannot mimic completely the response induced by mechanically injuring the leaves and applying caterpillar regurgitant. A specific elicitor in the regurgitant of the caterpillar enhances the amount of several systemically released volatiles. Thus, the systemic release of volatile compounds by herbivore-damaged cotton plants appears to be regulated by at least two different mechanisms.

**Keywords** Caterpillar regurgitant · *Gossypium* · Mechanical damage · *Spodoptera* · Systemic response · Volatiles

**Abbreviations** SPOD: *Spodoptera exigua* larvae · SYST-SPOD: Volatiles collected from undamaged upper leaves of the caterpillar damaged plant · SYST-ART: Volatiles collected from undamaged upper leaves of the artificially damaged plant · SYST-ART & REG: Volatiles collected from undamaged upper leaves of the artificially damaged plant with application of regurgitant · SYST-CTRL: Volatiles collected from undamaged upper leaves of an undamaged control plant

## Introduction

A number of plant species like corn, cotton, lima beans and cultivated tobacco are known to release volatile organic compounds (VOC) when under herbivore attack (Dicke et al. 1990; Turlings et al. 1990; Loughrin et al. 1994; De Moraes et al. 1998). This herbivore-induced release of volatiles may benefit the plant by attracting natural enemies of the herbivores that feed on its foliage and benefits parasitoids and predators by guiding them to potential hosts or prey on the plant (Dicke and Sabelis 1988; Turlings et al. 1991; Takabayashi et al. 1994; McCall et al. 1994). In addition, several of these compounds may be involved in direct defense against pathogens and herbivores (Caccioni et al. 1997).

The volatile compounds released from damaged cotton plants can be divided into constitutive compounds and inducible compounds. Constitutive compounds are

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released from damaged cotton leaves immediately after the beginning of feeding damage, or even after the plant is only artificially damaged with a razor blade to mimic the mechanical part of feeding damage (McCall et al. 1994; Röse et al. 1996). These early stages of plant damage are characterized by the release of "green leafy" volatiles like (*Z*)-3-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate and additional constitutive compounds that are plant specific (Turlings et al. 1995). Additional constitutive compounds in cotton are monoterpenes and sesquiterpenes (Loughrin et al. 1994) that are stored in lysigenous glands (Elzen et al. 1985). The constitutive terpenes in cotton are mostly cyclic and include α-pinene, β-pinene, limonene, (*E*)-β-caryophyllene, α-humulene and the acyclic monoterpene myrcene. After several hours of herbivore damage, plants start to release additional compounds that appear to be specifically released in response to herbivore damage (inducible compounds). Their release from the damaged leaves is not elicited in significant amounts by short-term artificial damage alone (Turlings et al. 1990; Röse et al. 1996). These herbivore-inducible compounds in cotton are acyclic terpenoids (i.e. (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene), (*Z*)-3-hexenyl acetate, indole, isomeric hexenyl butyrates, and 2-methylbutyrates (McCall et al. 1994; Loughrin et al. 1994).

Herbivore-induced compounds are not only released at the site of herbivore damage, but also systemically from the entire cotton plant (Röse et al. 1996). After several days of feeding by *Spodoptera exigua* larvae on the lower leaves of cotton plants, the upper undamaged leaves of the same plant released (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Röse et al. 1996). Cotton plants that are damaged on the lower leaves by *Helicoverpa zea* larvae release similar compounds systemically (Röse et al. 1998). However, in the field *H. zea* larvae prefer feeding on flower buds (squares) of cotton plants. Feeding damage to these reproductive structures of the plant causes a systemic release of volatiles from the vegetative parts of the plant (Röse and Tumlinson 2004). The compounds released systemically from leaves in response to feeding on flower buds were (*Z*)-3-hexenyl acetate, indole, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. The systemic release of volatiles in response to herbivory of different lepidoptera species on the lower leaves (Röse et al. 1998) or in response to herbivore feeding on flower buds (Röse and Tumlinson 2004) is very similar. This observation led us to investigate whether the systemic volatile release in response to herbivory is only the result of continuous mechanical damage of plant cells inflicted by the mouthparts of feeding caterpillars or whether it is a combined effect of the mechanical damage and herbivore-specific application of caterpillar oral secretion. An elicitor from oral secretion of regurgitating caterpillars induces for example corn seedlings to emit volatile compounds when applied to a mechanically damaged leaf (Turlings et al. 1993). The possibility to mimic caterpillar damage in this way was first observed by Turlings et al. (1990). Several hours after artificial damage with application of caterpillar regurgitant, those damaged leaves released large amounts of terpenoids, similar to the compounds induced by actual feeding of *S. exigua* larvae. Subsequently, an elicitor from oral secretion of *S. exigua* larvae was isolated and identified as *N*-[17-hydroxylinolenoyl]-*L*-glutamine (Alborn et al. 1997).

The ability to mimic caterpillar damage by applying caterpillar regurgitant to artificially damaged leaves is important, because it allows control of the amount of damage inflicted upon the leaves and the amount of regurgitant applied, both of which may vary with caterpillar feeding. This permits the observation of differences between an unspecific systemic wound response to mechanical damage and a specific response to caterpillar feeding damage or artificial damage plus application of caterpillar regurgitant.

We conducted experiments with intact plants to investigate whether a systemic response could be induced by different types of damage on the lower leaves of cotton plants and monitored the volatile emission over a period of 4 days. We determined how mechanical damage alone affected the systemic volatile release compared to the combined effect of mechanical damage plus application of caterpillar regurgitant. With this study we want to determine the specificity of a systemic volatile release in response to herbivory.

## Material and methods

### Plants

Approximately 6-week-old cotton plants, *Gossypium hirsutum* L. (Malvaceae), var. 'Deltapine acala 90′ (Delta and Pine Land Company, Hollandale, Scott, MS, USA), with eight fully developed leaves in addition to the two cotyledons, were used in the experiments. Cotton was grown in a greenhouse in a mixture of compost, peat moss and vermiculite (metro-mix 300, Scotts-Sierra Horticultural Company, Marysville, OH, USA) with natural light, under Florida summer conditions (14 L:10 D light cycle, 85 ± 10% RH, and 35 ± 10°C). Each cotton plant was grown from seed planted in a 16-cm-diameter pot and fertilized once at the time of planting with a 3–4 month formulation of Osmocote 14-14-14 (N-P-K) controlled release fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA).

### Lepidoptera larvae

Beet armyworm larvae (SPOD), *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were obtained from

the USDA rearing facilities in Gainesville, Florida. Larvae were reared on an artificial diet, based on pinto beans, according to the method of King and Leppla (1984). To encourage immediate feeding of larvae after being caged on the leaves, second and third instar larvae were starved for 12 h prior to the experiments. Each larva was confined in a separate cage that consisted of two halves of a modified Petri dish as previously, described (Röse et al. 1996) to define the area of feeding damage.

### Collection and processing of regurgitant from *S. exigua*

Third instar larvae were placed in a container and fed with corn leaves for 1 day before the regurgitant was collected. Regurgitation of the *S. exigua* larvae was induced by holding the caterpillars in the head region with a pair of light-weight forceps. The regurgitant was collected with a 100-µl pipette that was inserted in a 5-ml vial through a rubber septum. A second pipette was inserted through the septum and was connected to a low vacuum which allowed the regurgitant to drip slowly into the vial. About 15–20 µl regurgitant could be collected per larva. The crude regurgitant of several collection dates was stored in the freezer until a sufficient amount was collected. The regurgitant was then defrosted and pooled. One milliliter of crude regurgitant was pipetted into a centrifugation cup and centrifuged for 10 min. at 14,000 g in an Eppendorf Centrifuge (5415C). The supernatant was filtered through a 0.45-µm Millipore (Millex-GV) filter and through a 0.22-µm Millipore filter to remove bacteria. The regurgitant was stored at −70°C until used.

### *S. exigua* feeding damage

In previous experiments, we have observed a systemic volatile release from undamaged leaves in response to continuous feeding of two *S. exigua* larvae on each of two lower leaves of the same plant for several days (Röse et al. 1996). In this experiment, we determined if a systemic volatile release comparable to the one in response to continuous *S. exigua* damage could be induced by shorter feeding periods of twice the amount of caterpillars feeding on the lower leaves to synchronize it with the time of mechanical damage. Preliminary experiments had shown that a systemic response was not observed within the first few days if only two larvae were caged on each of the two lower leaves and removed overnight (Data not shown).

To induce a systemic volatile release, on day 1 at 12 p.m., four starved third-instar larvae were enclosed on each of two leaves, leaf five and six, of a cotton plant (counting from the youngest leaf) in a cage as previously described (Röse et al. 1996). The larvae were allowed to feed until 9 p.m. and subsequently removed from the leaves until the next morning. On day 2, new starved larvae were caged on leaf five and six and allowed to feed from 9 a.m. to 9 p.m. In the morning of day 3, the top four leaves of the plant were enclosed in a greenhouse volatile collection chamber as previously described (Röse et al. 1996) and remained in the collection system throughout the experiment. On day 3 and 4 larvae were caged on leaf seven and eight from 9 a.m. to 9 p.m. while volatiles were collected from the undamaged upper leaves (one-four) of the caterpillar damaged plant (SYST-SPOD).

### Mechanical damage

Cotton plants with eight true leaves were mechanically damaged with a plastic "derma-pik" (Greer Labs. Inc., Lenoir, NC, USA) which has 6 tines arranged in a circle of 2-mm diameter around the perimeter of the tip. The artificial damage started at the same time as the caterpillar damage in the SYST-SPOD treatment on day 1, from 12 p.m. to 9 p.m. and was continued on day 2 from 9 a.m. to 9 p.m. by punching leaf five and six for ten times per hour each with a "derma-pik". In the morning of day 3, the top four leaves of the plant were enclosed in a greenhouse volatile collection chamber. On day 3 and 4 leaves seven and eight were punched every hour from 9 a.m. to 9 p.m. and volatiles were collected from the undamaged upper leaves of the artificially damaged plant (SYST-ART).

### Artificial damage plus caterpillar regurgitant

The lower four leaves of the plant were treated to simulate mechanical damage (as described above). Prior to punching the leaf, each time the tip of the "derma-pik" was dipped into the caterpillar regurgitant applying a total of 20 µl of regurgitant per hour on both leaves at the sites of wounding. Volatiles were collected from the upper undamaged leaves of the damaged plant (SYST-ART&REG).

### Volatile collection

Volatiles were collected on day 3 and 4 from the undamaged upper leaves of SYST-SPOD, SYST-ART, SYST-ART&REG, and from undamaged control plants (SYST-CTRL) at times when partially damaged plants emitted a maximum of volatiles (12–3 p.m. and 3–6 p.m.; Loughrin et al. 1994). All plants were kept side by side in the greenhouse on day 1 and 2, with the control plant in the middle of the treated plants to detect any possible adsorption of volatiles to the top leaves.

To collect volatiles on day 3 and 4, the upper four leaves of each plant were enclosed in a volatile collection chamber that was part of an automated volatile collection system previously described (Heath and Manukian 1994; Röse et al. 1996). Two liters per minute of purified

air entered the system through the air diffuser inlet on top of the glass chamber. Volatile collector traps (150 mm long × 5 mm OD) containing 50 mg Super-Q as an adsorbent, were inserted in the side sampling ports located symmetrically around the base of the multiport guillotine base. Volatiles emitted from the upper portion of the cotton plant enclosed within the glass chamber were swept downward by the incoming pure laminar air flow. They were sampled at the bottom of the chamber by pulling air at a rate of 1 l/min through the volatile collection traps from a controlled vacuum source attached to each volatile collector trap from the automated volatile collection system. Thus, 50% of the air passed through the collector traps during the 3-h collection period, allowing for a higher detectability of small amounts of compounds systemically released. The remaining 50% excess air escaped through the opening at the bottom of the guillotine around the stem of the plant, preventing volatiles from the lower, damaged part of the plant from entering into the collection chamber containing the upper undamaged part of the plant.

### Analysis of volatiles

Volatiles were extracted from the collector traps by washing with 200 µl methylene chloride (capillary GC/GC-MS solvent, Burdick & Jackson, Muskegon, MI, USA). Internal standards were added (600 ng each of *n*-octane and nonyl acetate in 60 µl methylene chloride) to the extract. Samples were analyzed by gas chromatography (GC) and GC-mass spectroscopy. Of each collection sample, 1 µl was injected in the splitless mode on a bonded methyl silicone fused silica capillary column in a Hewlett–Packard gas chromatograph (model 5890 II plus) equipped with an auto injector (model 6890), a split–splitless capillary injector system and flame ionization detector (Röse et al. 1996). Helium at a linear flow velocity of 20 cm/s was used as a carrier gas. The temperature of the column oven was maintained at 40°C for 3 min, and then programmed at 5°C/min to 220°C, which was maintained for 10 min. The injector temperature was set at 220°C, the detector temperature at 260°C. Data collection, storage and subsequent analysis were performed on a Perkin Elmer chromatographic data system.

To identify compounds, volatiles were analyzed by GC-mass spectroscopy (GC-MS) with a Finnigan ITS-40 Magnum (ion-trap) mass spectrometer operated in electron impact and chemical ionization modes. For GC-MS the same fused silica capillary column and a DB5MS column (J&W Scientific, Folsom, CA, USA) were used with helium as a carrier gas, and for chemical ionization isobutane was used as reagent gas. Constituents of the plant volatiles were identified by comparison of mass spectra with spectra in the Environmental Protection Agency-National Institutes of Health database, the Environmental Protection Agency-National Institute of Standards and Technology database, and spectra obtained of authentic compounds. GC retention times of plant volatiles were also compared with GC retention times of those authentic compounds on the methyl silicone column, and the DB5MS column whenever they were available.

### Statistical analysis

Data were analyzed with the statistic program SYSTAT (Systat Inc., Evanston, IL, USA). Comparisons yielding a p-value $\leq 0.05$ were considered to be statistically significant. Each experiment was replicated six times. Since the amounts of the various volatiles released per plant frequently fell below detectable limits, the assumption that these amounts are normally distributed is unreasonable. Therefore, differences in the amounts of volatiles released per plant between SYST-SPOD, SYST-ART&REG, SYST-ART and SYST-CTRL leaves were analyzed nonparametrically. The Wilcoxon signed rank test was used to determine the significance of daily differences in volatile amounts between all four treatments separately for each replicate. The data were presented as box plots with a range of the central 50% of values and the median marked as a central horizontal line (Tukey 1977; Benjamini 1988).

## Results

The blend of volatiles released systemically on day 3 and 4 from the upper undamaged leaves of SYST-SPOD, SYST-ART&REG, SYST-ART, and SYST-CTRL contained the monoterpenes α-pinene, β-pinene, myrcene, (*E*)-β-ocimene, and linalool (Fig. 1), the sesquiterpenes (*E*)-β-farnesene and (*E,E*)-α-farnesene (Fig. 2), the homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Fig. 2), and (*Z*)-3-hexenyl acetate (Fig. 3). In addition, small amounts of indole and (*Z*)-jasmone were detected, depending on the treatment (Fig. 3).

After a total of 24–27 h of damage on the lower leaves (Fig.1, 2, 3, collection time A,), the SYST-SPOD, when compared to SYST-CTRL leaves, released significantly higher amounts of the monoterpenes β-pinene, (*E*)-β-ocimene and linalool (Fig.1A), the sesquiterpenes (*E*)-β-farnesene and (*E,E*)-α-farnesene (Fig. 2A), the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (Fig. 2A) and (*Z*)-3-hexenyl acetate (Fig. 3A) when compared to SYST-CTRL leaves. The observed differences between SYST-SPOD and SYST-CTRL leaves show that continuous damage as in previous experiments (Röse et al. 1996) was not necessary to induce a systemic response if twice the amount of caterpillars are caged on the leaves. Preliminary experiments had shown that a systemic response was not observed within the first few days if only two larvae were caged on each of the two lower leaves and removed overnight (Data not shown). The amount of damage appears to affect the

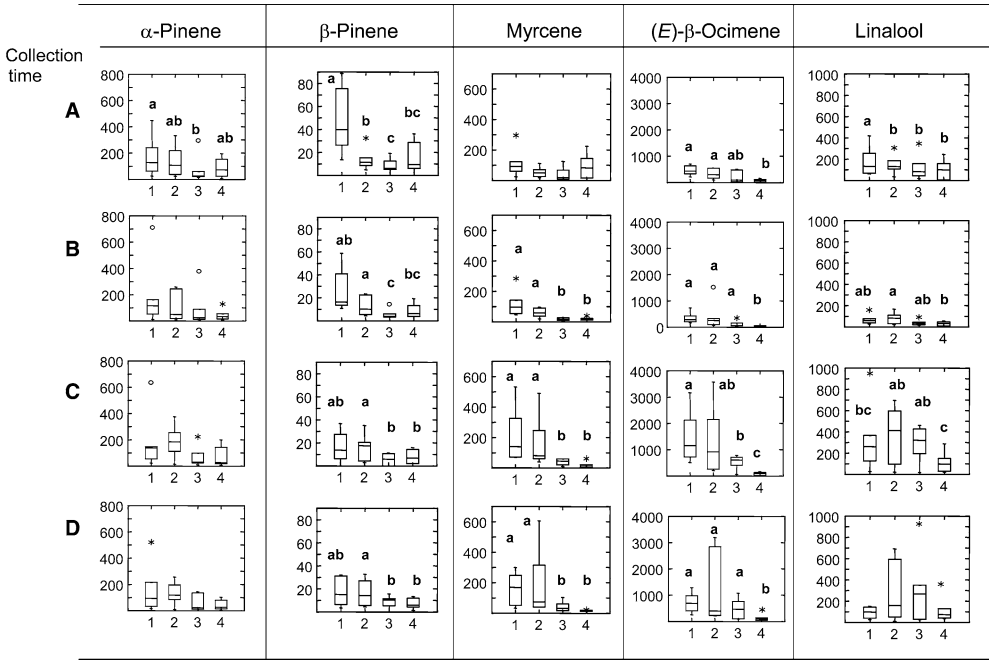


**Fig. 1** Amounts of different monoterpene volatiles (α-Pinene, *β*-Pinene, Myrcene, (*E*)-*β*-Ocimene, and Linalool) in nanograms of compound emitted systemically over 3 h per plant (*y*-axis of each graph) presented as box plots (*box* represents range of central 50% of values with median as a horizontal line over six replications). Volatiles were collected on day 3 from 12 p.m. to 3 p.m. (**A**) and from 3 p.m. to 6 p.m. (**B**) and on day 4 from 12 p.m. to 3 p.m. (**C**) and from 3 p.m. to 6 p.m. (**D**) from the upper undamaged leaves of four different treatments inflicted on the lower leaves (*x*-axis of each graph). *Lanes 1*, *S. exigua* damaged leaves (SYST-SPOD); *lanes 2*, leaves that were mechanically damaged with caterpillar regurgitant applied to the damage (SYST-ART & REG); *lanes 3*, mechanically damaged leaves (SYST-ART); *lanes 4*, undamaged control leaves (SYST-CTRL). *Different letters* indicate significant differences between the four treatments at one timepoint for one compound. *No letters* at one timepoint indicate no difference between the treatments. Values between the inner and outer fences (outliers) are plotted with *asterisks*; far outside values (extremes) are plotted with *empty circles*

systemic response but continuous overnight damage was not necessary for a systemic volatile release. However, at the same collection time A, SYST-ART&REG leaves released only significantly higher amounts of (*E*)-*β*-ocimene when compared to SYST-CTRL leaves (Fig. 1A), indicating that the response to our mechanical damage plus application of regurgitant was slower than the response to caterpillar feeding at comparable time intervals. The faster response of SYST-SPOD leaves may be explained by a higher amount of leaf area that was initially damaged by the caterpillars or a higher amount or concentration of regurgitant that was applied by uncontrolled feeding of caterpillars. Furthermore, caterpillars were allowed to feed continuously during the time interval on the leaves whereas the mimicking mechanical damage was inflicted only every hour. No differences were detected between the amounts of volatiles released from SYST-ART and SYST-CTRL leaves at that time.

On day 3 at the second collection time SYST-ART&REG leaves released significantly higher amounts of the monoterpenes *β*-pinene, myrcene, (*E*)-*β*-ocimene, and linalool (Fig. 1B), of the sesquiterpenes (*E*)-*β*-farnesene and (*E,E*)-α-farnesene, and of (*Z*)-3-hexenyl acetate when compared to SYST-CTRL leaves. Furthermore, SYST-ART leaves now released significantly higher amounts of (*E*)-*β*-ocimene and (*E,E*)-α-farnesene when compared to SYST-CTRL leaves. On day 4, after a total of 36–42 h of damage the monoterpenes *β*-pinene, myrcene, (*E*)-*β*-ocimene, and linalool (Fig. 1C,D), the sesquiterpenes (*E*)-*β*-farnesene and (*E,E*)-α-farnesene (Fig. 2C,D), the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (Fig 2C,D), and (*Z*)-3-hexenyl acetate (Fig. 3C,D) were released in significantly higher amounts from SYST-SPOD and SYST-ART&REG leaves when compared to SYST-CTRL leaves. No significant differences were observed between the amounts of volatiles released from SYST-SPOD and SYST-ART&REG leaves, indicating that the systemic volatile release induced by feeding damage of larvae on the lower leaves could be mimicked by artificially damaging those lower leaves and applying caterpillar regurgitant to the damage.

Besides a systemic volatile release in response to artificial damage plus application of caterpillar regurgitant, we also observed a systemic volatile release in response to artificial damage alone. The highest differences between SYST-ART leaves and SYST-CTRL leaves were observed on day 4 after a total of 36–39 h of artificial damage. SYST-ART leaves released significantly higher amounts of the monoterpenes (*E*)-*β*-ocimene and linalool (Fig 1C), the sesquiterpenes (*E*)-*β*-farnesene and (*E,E*)-α-farnesene and the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (Fig. 2C), and of

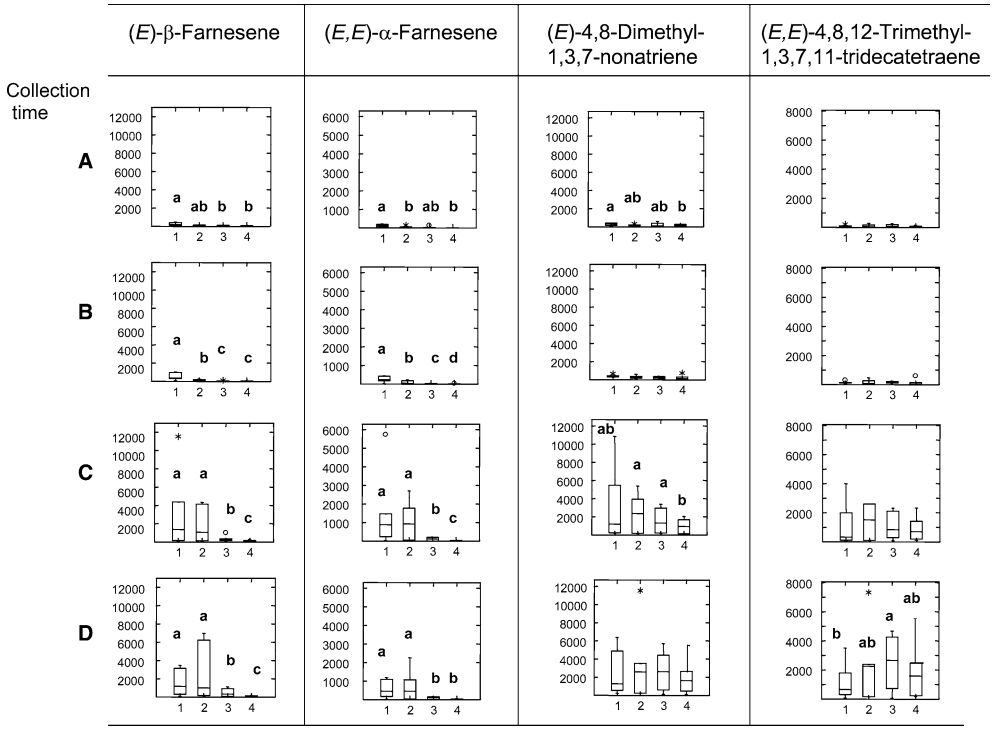


**Fig. 2** Amounts of different sesquiterpene volatiles ((*E*)-*β*-Farnesene, (*E,E*)-α-Farnesene)) and homoterpene volatiles ((*E*)-4,8-Dimethyl-1,3,7-nonatriene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) in nanograms of compound emitted systemically over 3 h per plant (*y*-axis of each graph) presented as *box plots* (*box* represents range of central 50% of values with median as a horizontal line over six replications). Volatiles were collected on day 3 from 12 p.m. to 3 p.m. (**A**) and from 3 p.m. to 6 p.m. (**B**) and on day 4 from 12 p.m. to 3 p.m. (**C**) and from 3 p.m. to 6 p.m. (**D**) from the upper undamaged leaves of four different treatments inflicted on the lower leaves (*x*-axis of each graph): *lanes 1*, *S. exigua* damaged leaves (SYST-SPOD); *lanes 2*, leaves that were mechanically damaged with caterpillar regurgitant applied to the damage (SYST-ART & REG); *lanes 3*, mechanically damaged leaves (SYST-ART); *lanes 4*, undamaged control leaves (SYST-CTRL). *Different letters* indicate significant differences between the four treatments at one timepoint for one compound. *No letters* at one timepoint indicate no difference between the treatments. Values between the inner and outer fences (outliers) are plotted with *asterisks*; far outside values (extremes) are plotted with *empty circles*

(*Z*)-3-hexenyl acetate and indole (Fig. 3C) when compared to SYST-CTRL leaves. When the amounts of volatiles released from SYST-ART leaves were compared to the amounts released from SYST-ART&REG leaves, clear differences were observed between those treatments even though the same amount of artificial damage was inflicted on the lower leaves of both plants at the same time. Differences were detected between the amounts of systemically released *β*-pinene, myrcene, (*E*)-*β*-farnesene, (*E,E*)-α-farnesene, and (*Z*)-3-hexenyl acetate (Fig. 1, 2, 3, treatments 2 and 3). As the only difference between the treatments was the application of regurgitant to artificially damaged SYST-ART&REG plants but not to SYST-ART plants, we can assume that it caused the systemic release of higher amounts of these compounds. However, no differences between SYST-ART&REG and SYST-ART leaves were observed for the amounts of systemically released (*E*)-*β*-ocimene and linalool (Fig. 1, treatments 2 and 3), and for the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (Fig. 2, treatments 2 and 3). It appears that the systemic release of these compounds can be elicited by artificial damage alone. No differences in the amounts of volatiles were observed between these treatments for (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene except on day four from 3 p.m. to 6 p.m. (Fig. 2D).

## Discussion

Natural enemies that attack a wide range of herbivorous insects, like the generalist parasitoid *Cotesia marginiventris*, can be innately attracted to both constitutive compounds released by short-term mechanical damage of cotton plants and to herbivore inducible compounds, while specialists like *Microplitis croceipes* are mainly attracted to the induced compounds (Röse et al. 1998). However, after the experience with the entire host–plant complex, these parasitoids in general prefer herbivore-induced volatiles. The defensive function of induced volatiles has been shown in field experiments (De Moraes et al. 1998; Bernasconi et al. 2001; Kessler and Baldwin 2001). For example, in *N. attenuata* plants the egg predation rates by a generalist predator increased and the oviposition rates of Lepidoptera decreased due to the presence of herbivore-induced volatiles (Kessler and Baldwin 2001). The presence of inducible defenses however is generally associated with metabolic costs that

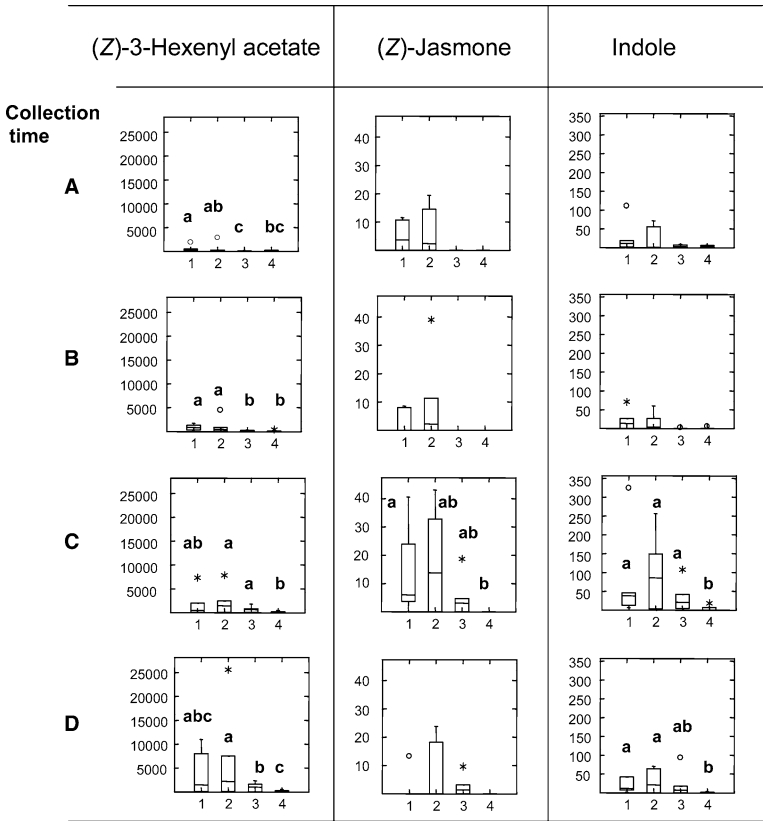


**Fig. 3** Amounts of different volatiles synthesized via the lipoxigenase pathway ((*Z*)-3-hexenyl acetate, (*Z*)-jasmone) and synthesized via the shikimic acid/tryptophane pathway (Indole) in nanograms of compound emitted systemically over 3 h per plant (*y*-axis of each graph) presented as box plots (*box* represents range of central 50% of values with median as a horizontal line over six replications). Volatiles were collected on day 3 from 12 p.m. to 3 p.m. (**A**) and from 3 p.m. to 6 p.m. (**B**) and on day 4 from 12 p.m. to 3 p.m. (**C**) and from 3 p.m. to 6 p.m. (**D**) from the upper undamaged leaves of four different treatments inflicted on the lower leaves (*x*-axis of each graph): *Lanes 1*, *S. exigua* damaged leaves (SYST-SPOD); *lanes 2*, leaves that were mechanically damaged with caterpillar regurgitant applied to the damage (SYST-ART & REG); *lanes 3*, mechanically damaged leaves (SYST-ART); *lanes 4*, undamaged control leaves (SYST-CTRL). *Different letters* indicate significant differences between the four treatments at one timepoint for one compound. *No letters* at one timepoint indicate no difference between the treatments. Values between the inner and outer fences (outliers) are plotted with *asterisks*; far outside values (extremes) are plotted with *empty circles*

result in the diversion of photosynthate from growth and reproduction to the synthesis of defense compounds (Gershenzon 1994; Kessler and Baldwin 2002). Terpenes are more costly to manufacture per gram than most other primary and secondary plant compounds (Gershenzon 1994). It is therefore not surprising that a one-time mechanical injury to a plant tissue may not elicit induced volatile defenses. This was observed in several studies that measured the volatile release in response to a single mechanical wounding event (Turlings et al. 1990; Röse et al. 1996; Halitschke et al. 2000). However, the application of oral secretion to a single mechanical wounding could elicit the release of inducible volatile compounds and thereby mimic herbivory (Turlings et al. 1990; Halitschke et al. 2000). For example, several hours after a single mechanical wounding with application of caterpillar regurgitant, corn leaves released large amounts of terpenoids, similar to the compounds induced by actual feeding of *S. exigua* on corn seedlings (Turlings et al. 1990). Furthermore, a systemic release of volatiles in response to one-time artificial damage with application of oral secretion of *S. exigua* larvae was observed for corn plants (Turlings and Tumlinson 1992) and for native tobacco plants after application of *Manduca sexta* oral secretion (Halitschke et al. 2000). In our study, cotton plants appeared to need a certain amount of herbivore damage on the lower leaves to respond with a systemic release of volatiles within the first 4 days. In a previous study, four third-instar caterpillars per plant were sufficient to induce a systemic volatile release if caterpillars were allowed to feed continuously (Röse et al. 1996), but not sufficient in this study if caterpillars were removed overnight. However, by doubling the number of caterpillars, we were able to induce a systemic release of volatiles, even when caterpillars were removed overnight. This indicates that a cotton plant may tolerate certain levels of herbivory before costly systemic inducible defenses are activated that can be detected as VOC in the headspace of a plant. The release of volatiles may depend on the level of wounding which is consistent with observations on *N. attenuata* plants (Halitschke et al. 2000).

With actual caterpillar feeding, the amounts of damage and applied oral secretion are very variable and

therefore different treatments are difficult to compare. With the ability to mimic caterpillar damage by applying regurgitant to artificially damaged leaves, we were able to observe differences between an unspecific systemic wound response to artificial damage and a systemic response to caterpillar feeding damage or artificial damage plus application of caterpillar regurgitant. In a previous study, volatiles released from an entire cotton plant mechanically damaged on its lower leaves every 2 h for a total of 51 h, and a plant similarly mechanically damaged with application of caterpillar regurgitant, showed that although mean levels of myrcene, (*E*)-*β*-ocimene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene were higher with application of regurgitant, significant differences between the two treatments were only detected for (*E*)-*β*-farnesene and (*E,E*)-α-farnesene and indole (Paré and Tumlinson 1997). Interestingly, several of these compounds were found to be synthesized de novo in response to caterpillar feeding. Caterpillar damaged cotton plants quickly incorporated $^{13}$C-labeled carbon dioxide within the first 30 h of exposure into (*E*)-*β*-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-*β*-farnesene, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Intermediate levels of $^{13}$C were incorporated in (*Z*)-3-hexenyl acetate and the monoterpenes limonene and myrcene, whereas α-pinene, *β*-pinene, (*E*)-*β*-caryophyllene, and α-humulene incorporated very low levels of $^{13}$C (Paré and Tumlinson 1997). Compounds that incorporated very low levels of $^{13}$C are stored in glands in the leaves and thus are present in relatively large amounts. Upon mechanical breakage of the glands these compounds are released immediately. Only (*Z*)-jasmone, a breakdown product of stored lipids of the lipoxygenase pathway, did not incorporate $^{13}$C.

While the release of herbivore inducible volatiles in cotton is clearly detectable from damaged leaves after 24–48 h, the systemic release of volatiles in cotton takes in general 3–4 days (Röse et al. 1996). Analysis of systemically released cotton volatiles in response to artificial damage (SYST-ART) and artificial damage plus regurgitant (SYST-ART&REG) revealed that regurgitant applied to the artificial damage was essential for a significantly higher systemic release of compounds like the monoterpenes *β*-pinene and myrcene, the sesquiterpenes (*E*)-*β*-farnesene and (*E,E*)-α-farnesene, and for (*Z*)-3-hexenyl acetate compared to only artificial damage in our experiments. The systemic release of significantly higher amounts of these compounds appeared to be triggered by an elicitor in the oral secretion of *S. exigua* larvae whereas the systemic release of several other compounds ((*E*)-*β*-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene) appeared to be triggered by mechanical wounding of the leaves alone. These results add further evidence to the active role of cotton plants in responding systemically to an elicitor in caterpillar oral secretion.

Oral secretion of regurgitating caterpillars contains an elicitor that induces corn seedlings to emit volatile compounds (Turlings et al. 1993) and induces cotton plants to systemically release certain volatile compounds in large amounts. An elicitor from oral secretion of *S. exigua* larvae was isolated and identified as *N*-[17-hydroxylinolenoyl]-*L*-glutamine (Alborn et al., 1997). The synthesized compound called volicitin induces the release of an identical blend of terpenoids and indole as *S. exigua* feeding on corn seedlings when applied to mechanically damaged leaves (Alborn et al. 1997). Structural similarities indicate that the elicitor may interact with the octadecanoid pathway or amplify the effect of mechanical damage by the larvae (Alborn et al. 1997). However, different plants and insects may have different mechanisms and a number of compounds that elicit a release of volatiles have been identified from different organisms. For example, oral secretion from cabbage feeding caterpillars of *Pieris brassicae* were reported to contain a *β*-glucosidase that induced volatile release in cabbage leaves (Mattiacci et al. 1995). And more recently, several fatty acid–amino acid conjugates isolated from regurgitant of different lepidopteran species have been identified (Paré et al. 1998; Pohnert et al. 1999; Alborn et al. 2000; Halitschke et al. 2001).

Furthermore, jasmonic acid that is produced by the octadecanoid signaling pathway is reported to induce the biosynthesis and release of volatile compounds (Hopke et al. 1994). Jasmonic acid is a damage-inducible compound in plants that appears to play an important role in the signaling of wound responses (Farmer and Ryan 1992; Sembdner and Parthier 1993; Creelman and Mullet 1995). For example, in tobacco plants, herbivory and mechanical damage both increased the concentration of jasmonic acid and the defense metabolite nicotine that is produced in the roots (McCloud and Baldwin 1997). While herbivory was reported to induce higher levels of jasmonic acid in the damaged leaves than mechanical damage, no differences between the treatments were reported for the induction of jasmonic acid and nicotine in the distant roots (McCloud and Baldwin 1997).

In summary, our results clearly show that a systemic volatile release in cotton can be induced by herbivory and by mechanical damage with application of caterpillar oral secretion to mimic herbivory but only to a lesser extent by mechanical damage alone. Multiple mechanical damage alone can not mimic completely the response induced by mechanically injuring the leaves and applying caterpillar regurgitant. Therefore, the systemic induction of volatile release is specific to herbivory. A specific elicitor in the regurgitant of the caterpillar enhances the amount of several systemically released volatiles. Thus, the systemic release of volatile compounds by herbivore damaged cotton plants appears to be regulated by at least two different mechanisms.

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